Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Orrú CD, Bongianni M, Tonoli G, et al. A test for Creutzfeldt–Jakob disease using nasal brushings. N Engl J Med 2014;371:519-29. DOI: 10.1056/NEJMoa1315200

SUPPLEMENTARY APPENDIX

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SUPPLEMENTARY METHODS

RT-QuIC data processing. To compensate for minor differences between fluorescence plate readers and baselines between individual samples, we performed a fluorescence baseline adjustment and normalized the baseline-adjusted values to percentages of the baseline-adjusted maximal fluorescence response from positive controls. Specifically, for each set of replicate reactions, the mean baseline rfu value was calculated over a 10h period spanning the lowest part of a plot of the mean rfu of all replicates versus reaction time. This value was then subtracted from the mean rfu values at each time point to give the baseline-adjusted mean rfu values ($\overline{rfu_{ba}}$). The latter were then normalized to give percentages of the maximum baseline-adjusted rfu value (i.e. 260,000 rfu-mean baseline rfu (Max_{ba}))

by dividing the baseline-adjusted rfu value by the baseline-adjusted maximum rfu value and multiplying by 100; i.e., $\left(\left(\frac{\overline{rfu_{ba}}}{Max_{ba}}\right) \times 100\right)$. These normalized values are plotted versus reaction time in Figures 1, S2-S4.

OM or CSF samples were judged to be RT-QuIC-positive using criteria similar to those previously described for RT-QuIC analyses of CSF specimens[1] except for the use of baseline adjusted, normalized fluorescence values and suitably adjusted cutoff values. Positive/negative assessments were made at the 50h and 90h time points, for the OM and CSF samples, respectively. The shorter time point was used for the OM samples for 2 reasons: 1) the higher seeding activity in the OM samples gave more rapid reactions, allow us to terminate the reactions much earlier without losing sensitivity, and 2) when the reactions were run longer than 55 h, on one occasion (1 out of >132 total reactions) negative control OM samples gave positive responses (which is not the case for CSF samples), suggesting that negative control OM samples can promote spontaneous (prion-independent) rPrP fibrilization after lag phases of >55h. A sample was considered positive if the mean of the highest two normalized fluorescence values from replicate wells (usually n=4) was higher than the mean from all negative control samples plus 10 standard deviations (for OM: 0.86 + 15.01 =15.87% max fluorescence; for CSF: -0.38 + 4.03 = 3.99%). To classify a positive sample two out of four wells must reach a value over the set threshold. In the event only three samples were run (in the case of some CSFs due to insufficient available sample volume) then the average of all three wells must be above threshold to be classified as positive.

Author Contributions

BC and GZ designed the study. GT perfomed nasal brushings set up the brushing method. GZ, CO, MB, GT, SF, AH, MF, and MP gathered, prepared and/or tested the samples. BC, GZ, CO, MB, SM, MP, BG, MF and AH analyzed the data and vouch for its analysis. BC and GZ wrote the paper, including the first draft,

with contributions and editing from MP, SM, CO and BG. There were no confidentiality agreements with sponsors.

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SUPPLEMENTARY FIGURES

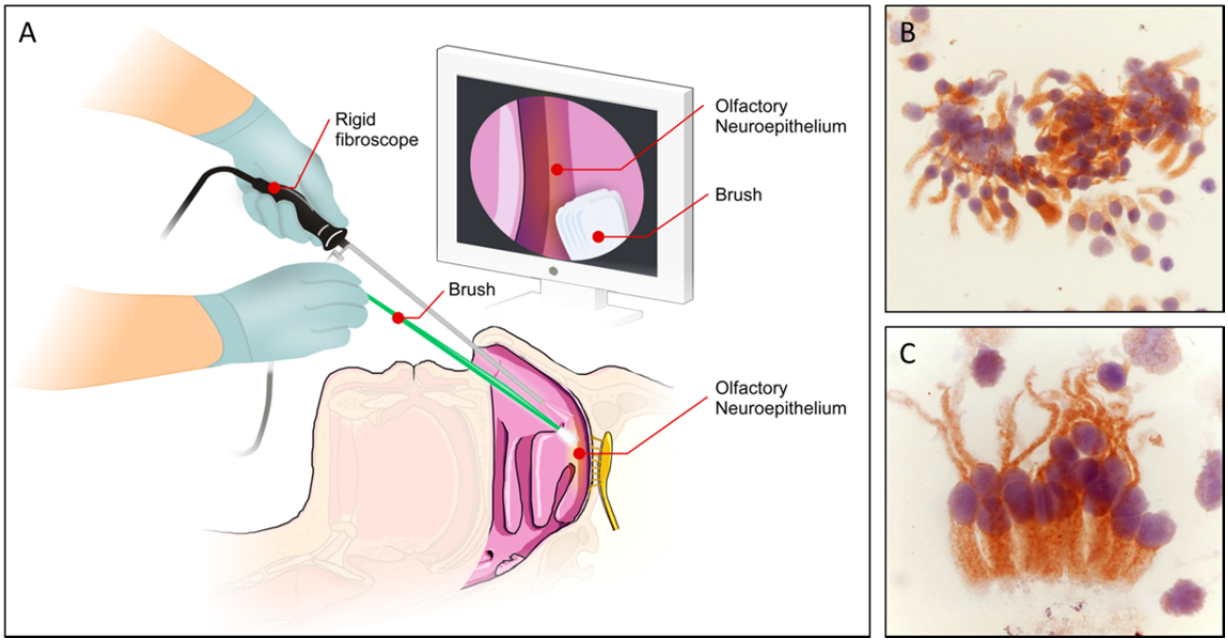

Figure S1. Olfactory mucosa brushing procedure. The operator inserts a rigid fiber-optic rhinoscope into the nasal cavity of the patient to locate the OM lining the nasal vault, easily distinguishable from respiratory mucosa because of its yellowish appearance. A sterile brush is inserted alongside the fibroscope and olfactory neurons are collected by gently rolling the brush on the mucosal surface (Panel A). Nasal brush cells obtained from two control subjects were immunostained with anti-olfactory marker protein (OMP) antibody, showing clusters of OMP positive olfactory neurons (Panel B, X 40 and panel C, X 100). An aliquot of OM pellet was immersed in 4% paraformaldehyde solution for 10min at room temperature and centrifuged for 6min at 700rpm in a cytocentrifuge. Cytocentrifuged preparations were stained either with hematoxylin and eosin to assess the cellularity or immunocytochemically by using a rabbit polyclonal antibody to olfactory marker protein (1:100, clone ab62144; Abcam, Cambridge, UK) to detect olfactory neurons.

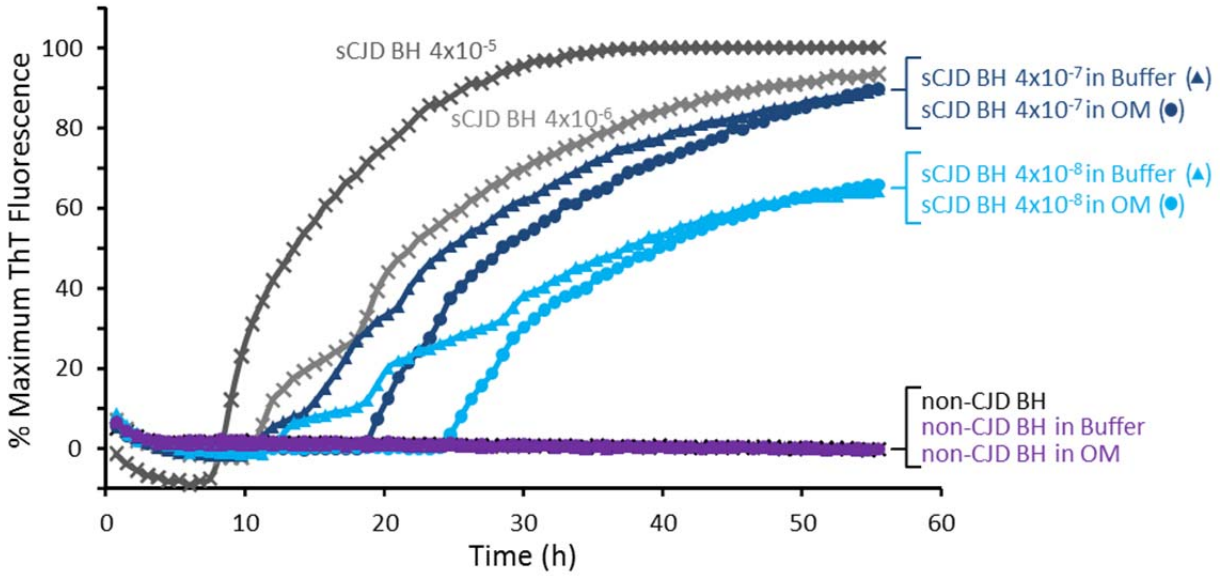

Figure S2. RT-QuIC detection of human sCJD brain homogenate spiked into non-CJD OM pellets. Sporadic CJD and non-CJD brain homogenate (BH) dilutions were diluted into human olfactory mucosa (OM) or buffer to give final dilutions of $4x10^{-7}$ and $4x10^{-8}$. Two µl containing ~10fg or 1fg of protease-resistant PrP^{CJD}, respectively, were used to seed RT-QuIC reactions as described in Methods. 10^{-5} and 10^{-6} dilutions of sCJD BH and a 10^{-6} dilution of non-CJD BH were used as positive and negative controls, respectively. The average % maximum ThT fluorescence from 4 replicate wells is shown as a function of reaction time.

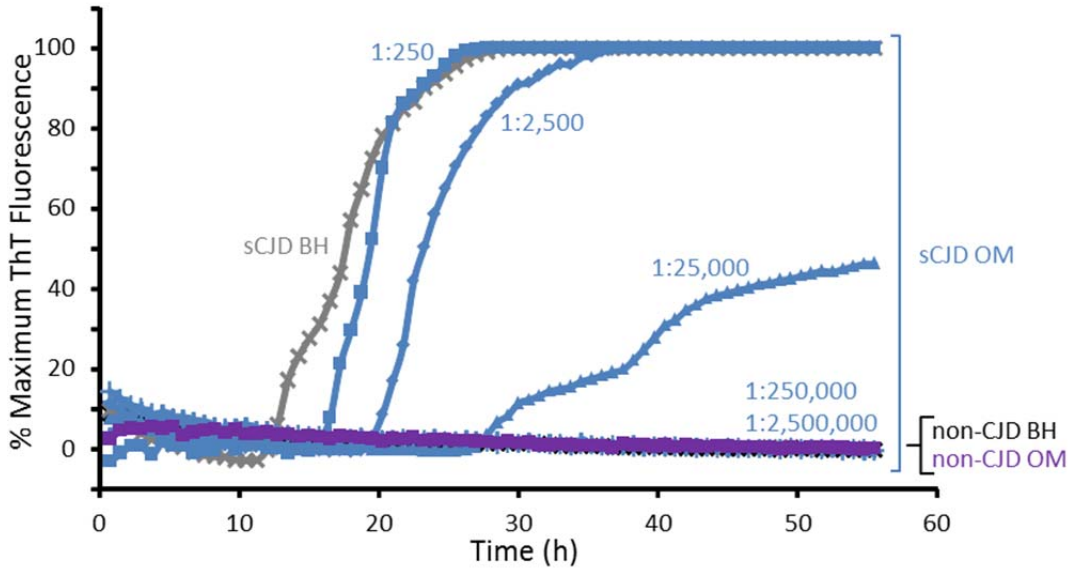

Figure S3. RT-QuIC end-point dilution analysis of sCJD OM. Two µl of the designated dilutions of an OM sample (Patient #7) were seeded into RT-QuIC reactions. Each time point is represented as an average % maximum ThT fluorescence from 4 replicate wells. A $4x10^{-6}$ dilution of sCJD BH (grey) was used as a positive control. The same dilution of a non-CJD BH (black) and a non-CJD OM (1:250; purple) were negative controls. The SD_{50} values of sCJD OM samples calculated for this and other patients are reported in Table 1.

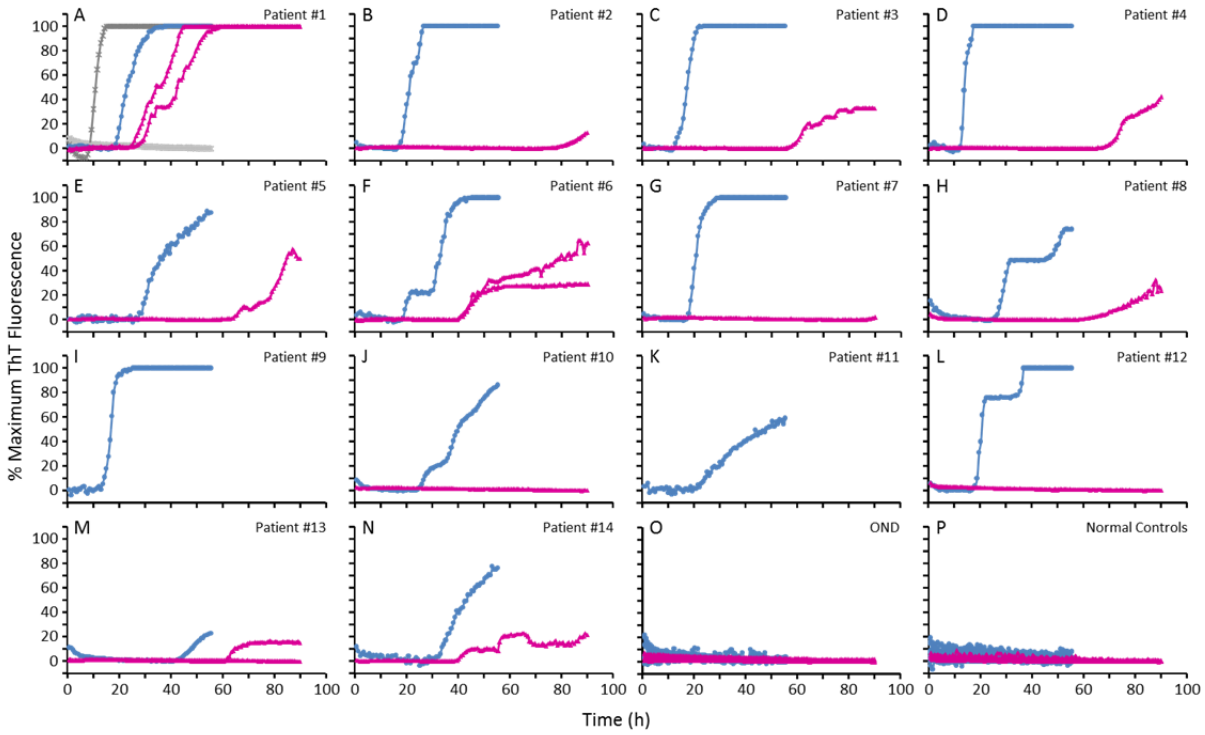

Figure S4. RT-QuIC comparisons of OM and CSF samples obtained from individual sCJD patients and from non-CJD controls. Twenty µl of neat CSF (magenta traces) and 1:250 dilutions of OM pellet (blue traces) samples obtained from 14 sCJD patients were tested in each reaction (Panels A-N). Forty three OM samples from non-CJD patients were also analyzed, including 12 patients with other neurodegenerative disorders (OND, Panel O) and 31 normal (non-neurological) controls (Panel P). Tests of CSF samples from 46 non-CJD controls, including 20 OND (Panel O) and 26 normal (non-neurological) controls (Panel P), are also shown. Serial dilutions giving final sCJD and non-CJD BH dilutions of 10^{-6} were used as positive and negative controls, respectively (dark and light grey traces, respectively, panel A). Patients # 1, 6 and 13 show two CSF traces resulting from two different lumbar punctures (for details see also Table 1). The vertical axis indicates the average % maximum ThT fluorescence from 3-4 replicate wells.

References

1. McGuire LI, Peden AH, Orru CD et al. RT-QuIC analysis of cerebrospinal fluid in sporadic Creutzfeldt-Jakob disease. Ann Neurol 2012;72(2):278-285.